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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF	:
TETSUYA SUGA, ET AL	: EXAMINER: BROOKS, KRISTIE LATRICE.
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DECLARATION UNDER 37 C.F.R. §1.132

COMMISSIONER FOR PATENTS
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SIR:

Now comes Yasuyo Suga, who declares and states that:

1. I am a graduate of University of Kyoto Institute of Technology, and received my bachelor degree in the field of agriculture, in the year 1991;

2. I have been employed by Ajinomoto Co., Inc., for 18 years as a researcher in R&D department involving in the study of medicine;

3. I have read and understand the present application and the Office Actions to date, including the references cited therein;

4. The following experiments were conducted by myself or under my supervision and control;

Experimental procedures

Preparation of β-glucan solution

Purification of β-glucan from raw shiitake mushrooms was carried out according to the method of Chihara et al. (Cancer Res., 30, 2776 (1970)). That is, fruit bodies of raw shiitake mushrooms were subjected to extraction with hot water, then repeatedly to fractionating precipitation with ethanol, to fractionating precipitation with cetyltrimethyl ammonium hydroxide, to fractionating elution with acetic acid and to fractionating elution with sodium hydroxide followed by removal of protein, whereby white powder of β-glucan was obtained. The resulting white powder was suspended in distilled water, homogenated and subjected to high-temperature and high-pressure treatment (121°C, 20 minutes) in an autoclave to prepare 2 mg/ml β-glucan solution (wherein β-glucan has an average diameter of about 200 μm).

Preparation of β-glucan solution of the present invention (Treatment of β-glucan with an emulsifier (emulsifying agent))

Lecithin (SLP-PC70) manufactured by Tsuru Lecithin Kogyo Co., Ltd. was added to deionized water to prepare a solution containing lecithin dissolved therein at a concentration of 8 mg/ml. To the lecithin solution was added the same volume of the above β-glucan solution, and the mixture was subjected to high-pressure emulsification treatment (emulsification pressure 1,500 kgf/cm^2) with a high-pressure emulsifier H11 model in a 2-step handle system, manufactured by Sanwa Kikai Co., Ltd., to prepare a micellar solution of β-glucan having a median diameter of about 100 nm (micellar β-glucan: the product of the present invention).

(* Measurement of the median diameter was carried out by a laser diffraction/scattering particle size distribution measurement method using an LA-910 particle size distribution meter manufactured by Horiba Seisakusho Co., Ltd.)

Evaluation of various β-glucan solutions for incorporation (absorption) into small intestinal Peyer's patch

The various samples (β-glucan solutions) prepared as above were evaluated for incorporation into small intestinal Peyer's patch according to the following method.

Preparation of XRITC-labeled β-glucan

The various β-glucan solutions prepared as above were labeled (XRITC-labeled) with XRITC AMINE (RESEARCH ORGANICS). That is, a solution comprising β-glucan converted into superfine particle or a non-adjusted β-glucan solution was prepared to make the β-glucan concentration 0.1 mg/ml, supplemented with 0.9 mM sodium metaperiodate up to 10 % of total volume, and stirred at 4 C for a whole day and night to subject β-glucan to oxidative cleavage. Subsequently, the solution obtained was supplemented with ethylene glycol up to 0.25 % of total volume to inactivate an unreacted sodium metaperiodate, and then dialyzed in distilled water at 4 C for a whole day and night. After completion of the dialysis, the solution was recovered, supplemented with XRITC AMINE solution in 1 μg/ml up to 20 % of total volume, adjusted to pH 9.5 with sodium hydroxide, and subjected to the reaction at 4 C for a

whole day and night while stirring. After the reaction, the reaction mixture was supplemented with sodium borohydride in 50 μg/ml up to 8 % of total volume for reduction, and subjected to the reaction at 4 C for a whole day and night while stirring. Finally, the reaction solution was adjusted to neutral pH with hydrochloric acid, and dialyzed at 4 C for a whole day and night or more. After completion of the dialysis, the solution recovered was concentrated using VIVASPIN 20, and the concentration was calculated by dividing the amount of β-glucan (which has been previously prepared) by the volume of the final concentrate for the use in the examination.

Observation of incorporation (absorption) from small intestinal Peyer's patch

Under ether anesthesia, male ddy mice (8 to 10 weeks old) were subjected to ventrotomy, and loops of 1 to 2 cm, each containing a Peyer's patch, were prepared in their small intestines. 0.1 ml of the solution comprising XRITC-labeled β-glucan converted into superfine particle or the solution comprising XRITC-labeled β-glucan (β-glucan consentration 0.25 mg/ml) was injected thereto. After an hour, sampling was conducted to prepare frozen sections. The frozen sections obtained were observed and photographed using fluorescence microscope.

Results of Evaluation

The results are shown in Figures 1 to 6.

Figure 1

Figure 2

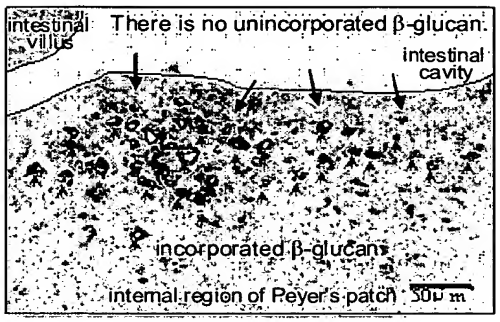


Figure 3

Figure 4

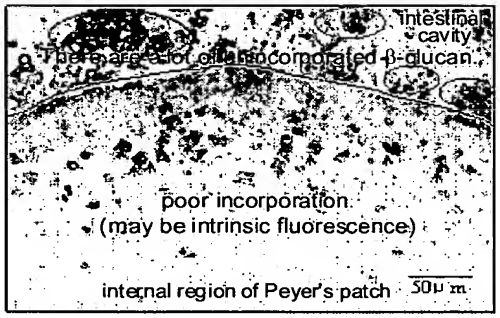


Figure 5

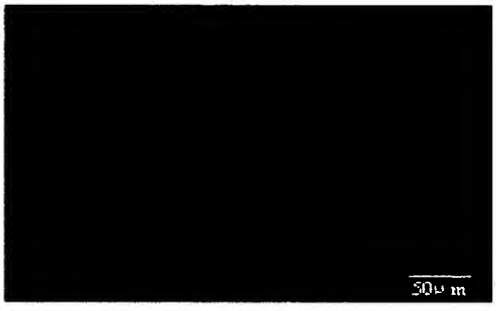
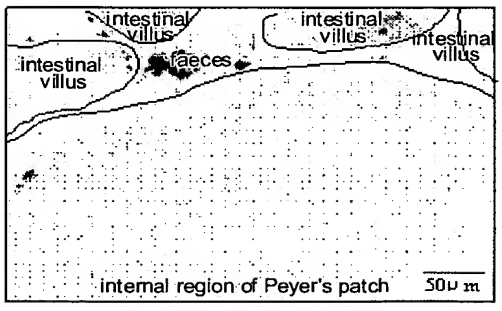


Figure 6



Absorbability of solution comprising β-glucan converted into superfine particle according to the present invention

In case of using (administering) β-glucan solution wherein the particle size was adjusted by converting β-glucan into superfine particle, fluorescence microscopy photograph (magnifying power: x 172) of small intestinal loop is shown in Figure 1, and a schematic diagram of the photograph is shown in Figure 2.

With regard to the photograph of Figure 1, as shown in Figure 2, there is no β-glucan in the intestinal cavity, and β-glucan is incorporated into the internal region of Peyer's patch. That is, it is found that the unincorporated β-glucan into the internal region of Peyer's patch is not observed, and all β-glucan (particles) are incorporated into the internal region of Peyer's patch.

Therefore, it is found that β-glucan which is converted into superfine particle to allow the average particle size to be smaller has a high absorbability.

Absorbability of non-adjusted β-glucan solution

In case of using (administering) the non-adjusted β-glucan solution (wherein β-glucan has an average diameter of about 200 μm), fluorescence microscopy photograph (magnifying power: x 172) of small intestinal loop is shown in Figure 3, and a schematic diagram of the photograph is shown in Figure 4.

With regard to the photograph of Figure 3, as shown in Figure 4, there is the poorly incorporated β-glucan into the internal region of Peyer's patch, and there is a lot of β-glucan in

the intestinal cavity. That is, it is found that a lot of unincorporated β-glucan into the internal region of Peyer's patch is not observed in the intestinal cavity, and a part of β-glucan (particles) are incorporated into the internal region of Peyer's patch.

Therefore, it is found that the non-adjusted β-glucan has a poor absorbability.

Control

In case that β-glucan solution is not administered to small intestinal loop (in case of control), fluorescence microscopy photograph (magnifying power: x 172) of small intestinal loop is shown in Figure 5, and a schematic diagram of the photograph is shown in Figure 6.

In the photograph of Figure 5, there is the region of Peyer's patch at the bottom, and there is the intestinal cavity at the upper part thereof. There are faeces and internal villus in the intestinal cavity.

Conclusion

From these results, it is found that a large amount of superfine particles (β-glucan) of the present invention are absorbed in small intestinal Peyer's patch, that is, β-glucan converted into superfine particle according to the method of the present invention has a high absorbability.

Additionally, these results provide the following consideration:

Unexpected results:

(1) The stabilized fine particle can be prepared from the aggregate by treating β-glucan with lecithin under high temperature and high pressure.

(2) The exertion of an inhibitory effect on tumor growth is dependent on a particle diameter. The critical point is 10 μm, and such effect is produced in case of a particle diameter of 10 μm or less.

(3) β-glucan converted into superfine particle is absorbed in small intestinal Peyer's patch, whereas β-glucan, which is not converted into superfine particle, is not absorbed in small intestinal Peyer's patch.

(4) β-glucan converted into superfine particle is absorbed in small intestinal Peyer's patch to produce an inhibitory effect on tumor growth.

Reasons why these results would be considered unexpected:

(1) For improving absorbability of a substance having a high molecular weight and poor absorbability, such as β-glucan, a method for converting into low molecular weight substance is generally selected.

It is a novel idea that absorbability is poor due to large particle diameter in solution, and thereby such effect is not produced.

There are no reports of a method for enhancing absorbability through a step of converting into superfine particle (instead of a step of converting into low molecular weight substance) to produce such effect, so far.

(2) There are no reports of β-glucan, wherein intestinal absorbability is enhanced to produce such effect, so far.

5. I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

6. Further Declarant saith not.

Yasuyo Suga

Yasuyo Suga

2009. 09. 07

Date